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# The boundary of macaque rDNA is constituted by low-copy sequences conserved during evolution

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**Abstract**

In *Macaca mulatta,* the single rDNA array is flanked by a patchwork of sequences including subregions of human Yp11.2, 4q35.2, and 10p15.3. This composite DNA region is characterized by unique or low-copy sequences, resembling a potentially transcribed region. The analysis of *Cercopithecus aethiops, Presbytis cristata,* and *Hylobates lar* suggests that this complex sequence organization could be shared by Old World monkey and lesser ape species. After the lesser apes/great apes divergence, the unique or nonduplicated DNA region underwent amplification and spreading, preferentially marking the p arm of acrocentric chromosomes bearing the rDNA. The molecular analysis of human acrocentric chromosomes revealed some extent of remodeling of the rDNA boundary: near the human NOR, a large 4q35.2 duplication partially resembles that found in MMU; conversely, infrequently represented Yp11.2 sequences totally differed from those of the macaque, and 10p15.3 sequences were lacking. Thus, although evolutionary events modified the sequence organization of the MMU rDNA boundary, its overall sequence feature and the preferential location in vicinity to the NOR have been conserved.


*Keywords:* Low-copy sequences; Duplication; rDNA boundary; Primate evolution; *FRG1*

The p arms of human acrocentric chromosomes are composed of three cytogenetic bands: p11, p12, and p13 (ISCN 1995). In the p12 band, acrocentric chromosomes carry an uninterrupted array of ribosomal DNA repetition units [1–3], flanked on both sides (p11 and p13 bands) by nonribosomal DNA. Fluorescence in situ hybridization (FISH) experiments have been extensively used to investigate the sequence content of p11 and p13 bands. The proximal (centromeric) band contains several types of tandemly highly repeated DNA, including classical satellite DNAs I, II, and III [4,5], β-satellite sequences (BSR), and LSau sequences, as a part of a 3.3-kb larger repetition (D4Z4-like sequences) [6–11]. Furthermore, FISH studies with human BACs deriving from 15p and 22p evidenced on the short arm of human acrocentrics the occurrence of duplicated sequences 4q24 and 4q35.2 [12,11].

Recently, we identified a duplication of approximately 60–80 kb carrying the prototype of BSR, preferentially localized on the short arms of human acrocentric chromosomes [13]. The ancestral locus of this sequence is a chromosomal region close to the rDNA on the marker chromosome (chromosome 13) of the macaque (*Macaca mulatta;* MMU). In great apes, this sequence underwent duplication and spreading involving, among others, the chromosomal regions known to bear rDNA genes. Like many pericentromeric regions, p arms of acrocentric chromosomes are generally regarded as heterochromatic zones into which expressed (i.e., ribosomal DNA) genes are embedded, thus representing a model to explore heterochromatin/euchromatin transition.

Currently, few data are available on the types of sequences (highly repeated vs unique or low-copy sequences) flanking rDNA in primates. The lack of data is due to several concurrent reasons, including sequence-assembling difficulties inherent in the repetitive nature of these domains, lack of appeal because of the absence of functional genes other than rDNA, and presence

*Abbreviations:* BSR, β-satellite repeat; rDNA, ribosomal DNA.

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of highly variable polymorphisms (some of them cytogenetically detectable) complicated by the high similarity, in humans, among all the NOR-bearing chromosomes.

In this paper we report studies on the organization of the rDNA domain in macaque. The macaque was chosen because (i) a BAC library for this species is available, (ii) a sequencing project for this species is in progress, (iii) our previous studies suggest that the MMU rDNA–non-rDNA organization represents a lower status of complexity with respect to humans [13], and (iv) rDNA in MMU is localized on a single chromosome, thus reducing analysis complexity.

Our study allowed the definition of a peculiar sequence organization characterized by the occurrence on both sides of the MMU rDNA array of a patchwork of unique and low-copy sequences. This feature was conserved during evolution, characterizing the short arm of great ape and human acrocentric chromosomes.

## Results

### *Isolation of MMU genomic clones at the boundary of rDNA*

A MMU genomic library was screened using a DNA probe (identified as 2* in Fig. 1 and as Yp11.2 (2*) in Supplementary Tables 1 and 2) corresponding to a human sequence located downstream of the BSR prototype carried on a Yp11.2 duplication [13]. Two of the isolated BAC clones (CH250-26D6 and CH250-50J16), when hybridized in situ to MMU metaphases, detected only the secondary constriction of chromosome 13, harboring the rDNA (Fig. 2 and Table 1). The use of the same clones on stretched MMU metaphases revealed split signals closely flanking the secondary constriction. Fig. 2 (inset) reports the hybridization pattern obtained by the clone 50J16. These results demonstrated the presence of similar sequences on both sides of the MMU rDNA gene cluster.

### *Characterization of DNA sequences at the boundary of MMU rDNA*

We then characterized BAC CH250-50J16 by partial sequencing. In the human genome databank, the derived BAC ends and inter-*Alu* sequences matched rDNA (U13369), 4q35.2 (AF146191), and 10p15.3 (AL359957) regions (Supplementary Table 2). To investigate further the sequence composition of the MMU clone we carried out paralogous polymerase chain reactions with primer pairs designed on the human detected regions: rDNA (clone U13369; primer pairs 18S, 28S, and IGS-1), 4q35.2 (clone AF146191; primer pairs a–d), and 10p15.3 (clone AL359957; primer pair α). Primer pairs were also designed on clone AC006987 (Yp11.2; primer pairs 1–4 and BSR), harboring the sequence used for the screening of the MMU genomic library. Primer sequences and their locations on

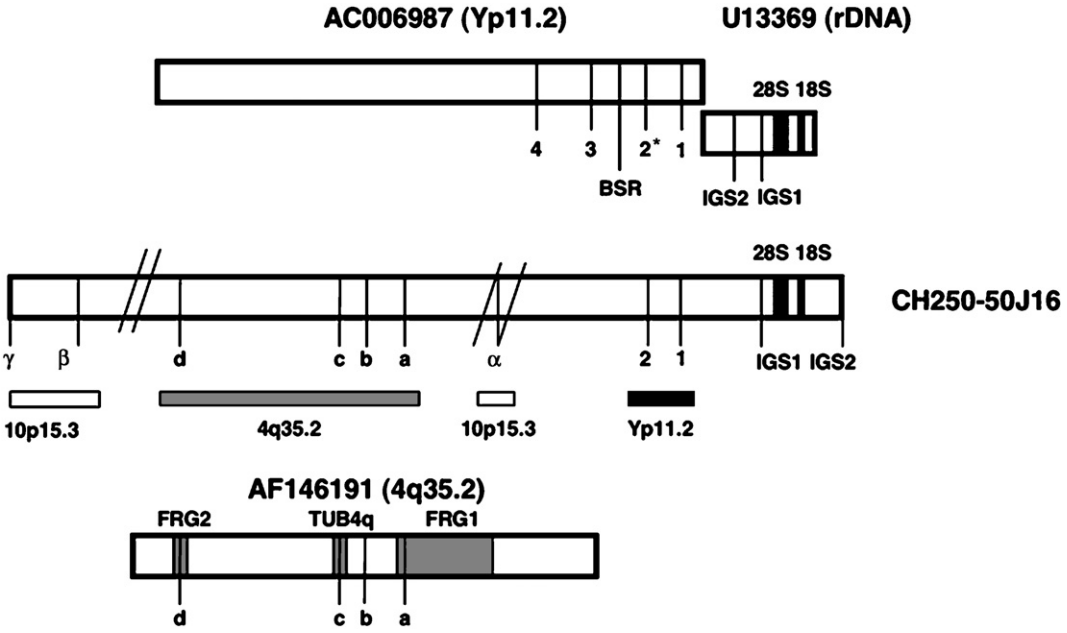


Fig. 1. Schematic sequence organization of the MMU BAC CH250-50J16 in comparison to human DNA regions located on chromosomes Yp11.2 (AC006987) and 4q35.2 (AF146191) and to the rDNA unit (U13369). The sequence organization was derived by end and internal sequencing and by paralogous polymerase chain reaction carried out using a set of primer pairs designed on human reference sequences U13369, AC006987, AF146191, and AL359957 (10p15.3). The position of each primer pair is identified by thin vertical bars labeled IGS1, IGS2, 18S, and 28S (rDNA); 1–4 and BSR (Yp11.2); a–d (4q35.2), and α (10p15.3). The β-and γ-labeled bars identify, respectively, the positions of sequences corresponding to one *Alu*-polymerase chain reaction and to the T7 end. Additional information for the DNA sequences derived by the analysis of BAC CH250-50J16 is reported in Supplementary Table 2. The DNA region identified by 2* (Yp11.2) represents the human DNA probe used for the screening of the MMU genomic library. Thick vertical bars identify the positions of 18S and 28S (solid black) and FRG1, FRG2, and TUB4q (solid gray). Horizontal bars identify the approximate localization of each genomic region within the MMU BAC CH250-50J16: 4q35.2 (gray), 10p15.3 (empty), and Yp11.2 (black). The MMU clone contains at the SP6 end one almost complete rDNA unit linked to a region enriched in interspersed repeats (SINE and LINE) and genes and/or pseudogenes.

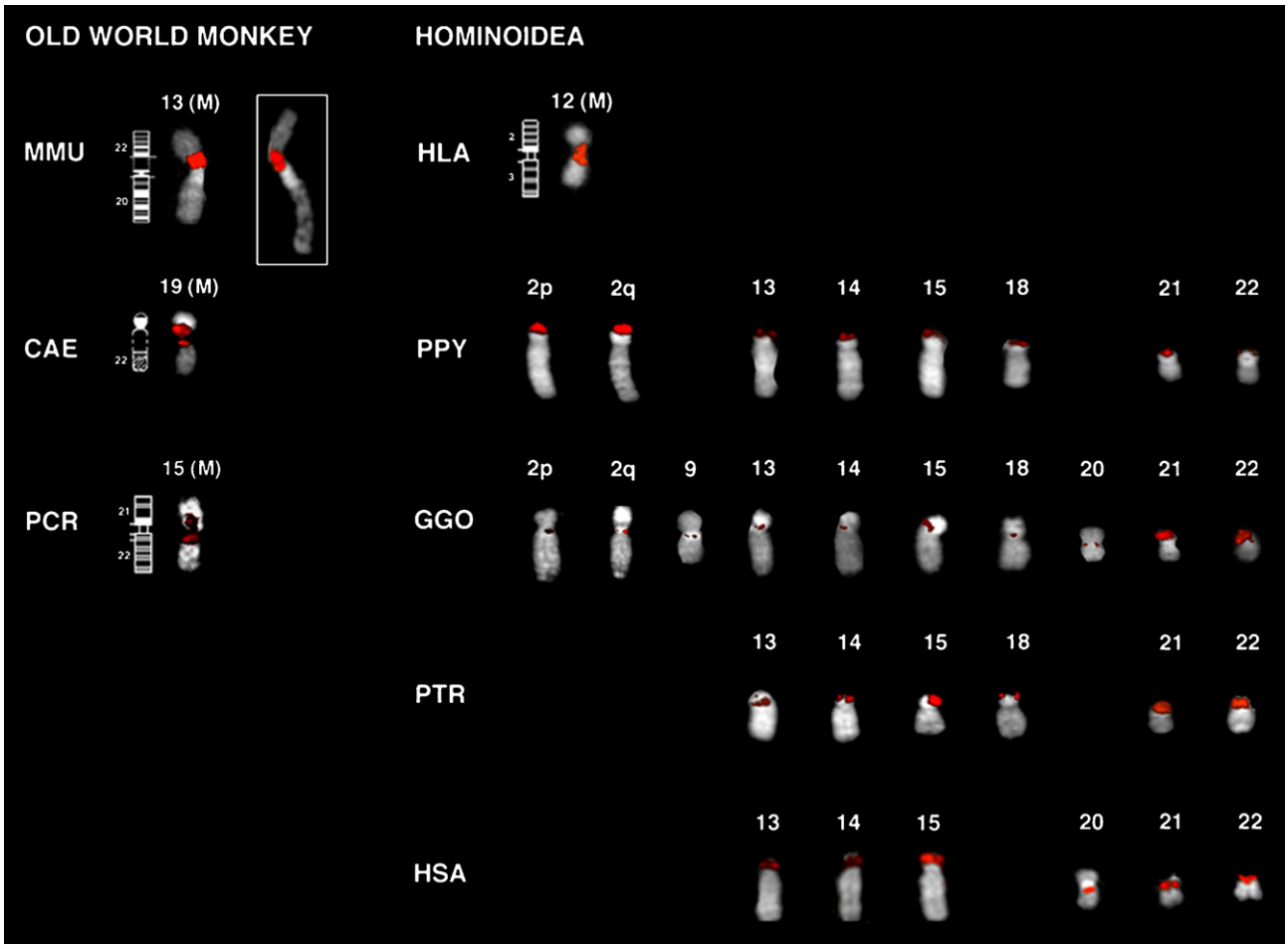


Fig. 2. Chromosome locations on primate species of MMU sequences found at the boundary of rDNA. Comparative FISH experiments were carried out using as probe the MMU 50J16 BAC on Old World Monkey (OWM) (*Macaca mulatta,* MMU; *Cercopithecus aethiops,* CAE; and *Presbytis cristata,* PCR) and hominoid (*Hylobates lar,* HLA; *Pongo pygmaeus,* PPY; *Gorilla gorilla,* GGO; *Pan troglodytes,* PTR; and *Homo sapiens,* HSA) metaphase chromosome spreads. For the OWM and HLA species, the reported chromosome (MMU-13, CAE-19, PCR-15, and HLA-12) is the only hybridizing chromosome, and the idiograms on the left depict the corresponding syntenic human chromosomes. Marker chromosomes MMU-13(M), CAE-19(M), PCR-15(M), and HLA-12(M), the only chromosomes of the analyzed species bearing the rDNA array, are derived by the fusion of chromosomes 20 and 22, 22 and unknown DNA, 21 and 22, and 2 and 3, respectively. The inset shows the split hybridization pattern obtained by the MMU 50J16 probe on the stretched MMU-13 chromosome. Except for OWM and HLA species, Arabic numbers refer to the homologous human chromosome.

human reference clones can be derived from Supplementary Table 1.

By this approach we derived the general organization of the MMU BAC 50J16, which contained at least 60–70 and 15–20 kb of 4q35 and Yp11.2 regions, respectively, linked to rDNA (Supplementary Table 2 and Fig. 1). By Southern blot hybridization we confirmed the occurrence of 18S and 28S rDNA genes and the absence of α-satellite DNA (not shown). The alignment of the 43-kb rDNA unit (U13369) to CH250-50J16-Sp6 and the occurrence, in the BAC, of both 18S and 28S units, allowed us to establish the orientation of rDNA genes (Fig. 1).

The same analysis carried out on the second MMU BAC (CH250-26D6), which evidenced essentially the same features of 50J16. The ends of the second BAC are included within the 50J16 sequence (Supplementary Table 2) and the sequenced regions shared by the two BACs showed a similarity of 100%, thus strongly suggesting that CH250-26D6 is completely contained within BAC CH250-50J16 (not shown).

### *Evolution of DNA sequences at the boundary of the rDNA cluster*

To investigate the evolutionary history of the sequences flanking the rDNA in the macaque genome, we hybridized the MMU 50J16 clone to primate chromosome spreads (*Cercopithecus aethiops* (CAE), *Presbytis cristata* (PCR), *Hylobates lar* (HLA), *Pongo pygmaeus* (PPY), *Gorilla gorilla* (GGO), *Pan troglodytes* (PTR), and *Homo sapiens* (HSA)). Similar to the macaque, the CH250-50J16 clone yielded split signals only around the rDNA clusters in *C. aethiops* (CAE chromosome 19) and *Pr. cristata* (PCR chromosome 15) (Fig. 2 and Table 1). Also in the gibbon (HLA) the probe highlighted the single NOR, located at the secondary constriction of chromosome 12. Conversely, the other analyzed species (PPY, GGO, PTR, and HSA) showed multiple chromosome locations that always included all the chromosomal regions known to bear the rDNA (Fig. 2 and Table 1). Particularly in PPY, PTR, and HSA the distribution of hybridization signals was almost

Table 1
Summary of FISH analysis carried out by MMU and HSA BACs on primate chromosome spreads

| Species | BAC probe | Chromosomes | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MMU | 26D6 | | | | | | | | | | | | **13M** (20–22) | | | |
| | 50J16 | | | | | | | | | | | | **13M** (20–22) | | | |
| | AF146191 | 1 | | 3 | 4qtel | 5 | | | | | | | **13M** (20–22) | | | |
| | AC006987 | No signals | | | | | | | | | | | | | | |
| | AL359957 | | | | | | 10ptel | | | | | | | | | |
| PCR | 50J16 | | | | | | | | | | | | **15M** (21–22) | | | |
| | AF146191 | | | 3 | 4qtel | | | | | | | | **15M** (21–22) | | | |
| CAE | 50J16 | | | | | | | | | | | | **19M** (22) | | | |
| | AF146191 | | 2 | | 4qtel | | 9 | 10 | | | | | **19M** (22) | | | |
| HLA | 50J16 | | **12M** (2–3) | | | | | | | | | | | | | |
| PPY | 50J16 | | **2p, 2q** | | | | | | **13,14,15** | | **18** | | **21,22** | | | |
| GGO | 50J16 | | 2p, 2q | | | | 9 | | 13,14,15 | | 18 | 20qcen | **21,22** | | | |
| PTR | 50J16 | | | | | | | | **13,14**,15 | | **18** | | **21,22** | | | |
| HSA | 50J16 | | | | | | | | **13,14,15** | | | 20qcen | **21,22** | | | |
| | AF146191 | | | | 4qtel | | | 10ptel–qtel | **13,14,15** | | | 20qcen | **21,22** | | | |
| | AC006987 | | | | | | | | **13,14,15** | | | 20qcen | **21,22** | | X | Yp–q |
| | AL359957 | | | | | | | 10ptel | | | | | | | | |

OWM species (*Macaca mulatta,* MMU; *Presbitys cristata,* PCR; *Cercopithecus aethiops,* CAE), a lesser ape (*Hylobates lar,* HLA), great apes (*Pongo pygmeus,* PPY*; Gorilla gorilla,* GGO; *Pan troglodytes,* PTR), and human (HSA) chromosomes spreads were hybridized with MMU BACs 50J16 and 26D6 and with HSA BACs AF146191 (4q35.2), AC006987 (Yp11.2), and AL359957 (10p15.3). Except for marker chromosomes of MMU (13M), PCR (15M), CAE (19M), and HLA (12M), primate chromosomes are indicated as the human homologous chromosomes. Chromosomes MMU-13, PCR-15, CAE-19, and HLA-12 are derived, respectively, by the fusion of chromosomes 20 and 22, 21 and 22, 22 and unknown DNA, and 2 and 3 (in parentheses). Chromosomes carrying the rDNA array are listed in boldface.

comparable to that of the rDNA arrays, with very few additional loci, such as chromosomes 15 and 20 in PTR and HSA, respectively. The gorilla, on the contrary, showed a very widespread distribution of 50J16 sequences in comparison to the restricted location of rDNA clusters on only two chromosomes (21 and 22). However, the intensity of hybridization signals was significantly higher on the chromosomes bearing rDNA arrays than on the other chromosomes, which included 2p, 2q, 9, 13–15,18, 20 (Fig. 2 and Table 1). On great apes and human chromosomes few grains were occasionally detected at the 4q region. The detection by the 50J16 probe of the great apes and human 4qtel, but not of the Old World Monkey (OWM) homologous region, may suggest the occurrence of some reorganization of this region during evolution. Moreover, comparative FISH experiments on primate chromosomes indicated that 50J16 sequences underwent duplication and spreading after the separation of OWM from the great ape–human lineage.

To investigate further the evolution of sequences found at the boundary of rDNA in the macaque genome, OWM and HSA chromosome spreads were also hybridized with human BACs representative of genomic regions 4q35.2, Yp11.2, and 10p15.3 (BACs AF146191, AC006987, and AL359957, respectively), part of which are contained within MMU 50J16 sequences. In the OWM species, the human 4q35.2 probe (AF146191) gave signals on multiple chromosomes (Fig. 3A and Table 1), always including the 4q ancestral locus and the chromosome carrying the rDNA cluster (MMU-13, CAE-19, and PCR-15) (Fig. 3A). Particularly, in the macaque the 4q35.2 probe (AF146191) decorated both sides of the rDNA locus, whereas in CAE and PCR only one side of the rDNA cluster was detected. The comparison between the hybridization pattern produced by BACs MMU 50J16 and HSA AF146191 clearly shows that on the boundary of the OWM rDNA cluster, each probe highlighted a distinct sequence block: 4q35.2 sequences are located more distal to the NOR than 50J16 sequences (compare Figs. 2 and 3A). Conversely, the hybridization of the probes representative of human Yp11.2 (AC006987) and 10p15.3 (AL359957) on MMU chromosomes gave no hybridization signal and a single hybridization spot at the human homologous 10ptel, respectively (Table 1). These findings suggested that Yp11.2 and 10p15.3 sequences are underrepresented in the MMU genomic regions identified by the 50J16 probe and that the OWM genome does not carry extensive duplication of 10ptel and Yp11.2 regions. On human chromosomes, 4q35.2 and Yp11.2 probes originated a spreading pattern almost comparable to that shown by 50J16 sequences, comprising mainly the short arms of acrocentric chromosomes and 20qcen (Fig. 3B and Table 1). Additional detected loci were 4qtel/10ptel–qtel and Yp–q/Xcen for 4q35.2 and Yp11.2 probes, respectively (Fig. 3B and Table 1). Conversely, the 10ptel probe detected only the corresponding locus (Table 1). The FISH experiments with AF146191 and AC006987 human probes clearly indicated that the duplication and spreading of 4q35 and Yp11.2 sequences took place at different times, before and after the great ape/OWM divergence.

Since on the short arm of human acrocentric chromosomes the 4q35.2 and Yp11.2 sequences showed a superimposable hybridization pattern, the AF146191 and AC006987 probes were cohybridized to stretched chromosomes obtained from a somatic cell hybrid carrying only chromosome 22 or 15 as a human content, to map them precisely in relation to the human rDNA cluster (Fig. 4). On both acrocentrics, 4q35.2 signals (red) were more proximal to the rDNA cluster (green signals) than Yp11.2 sequences (blue signals). A very similar result was obtained by the cohybridization of Yp11.2 and 50J16 BACs (not shown).

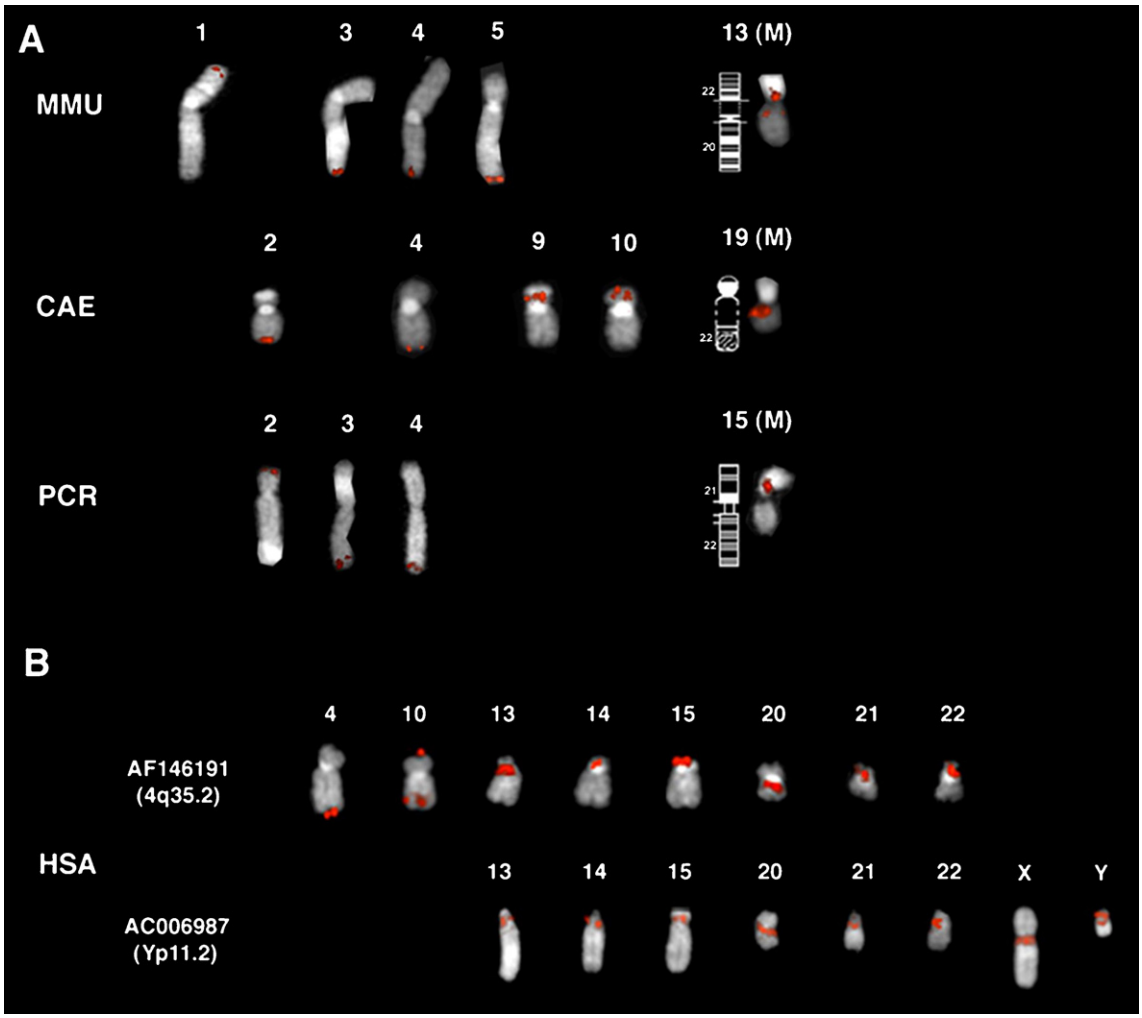


Fig. 3. Chromosome locations on primate species of human 4q35 and Yp11.2 sequences. (A) Comparative FISH experiments were carried out using BAC HSA AF146191 on Old World Monkey (*M. mulatta,* MMU; *C. aethiops,* CAE; and *Pr. cristata,* PCR) metaphase chromosome spreads. Except for the marker chromosomes (MMU-13, CAE-19, and PCR-15), the only chromosomes of the analyzed OWM species bearing the rDNA array, Arabic numbers refer to homologous human chromosomes. The idiograms on the left of the marker chromosomes depict the corresponding syntenic human chromosomes. MMU-13(M), CAE-19(M), and PCR-15 (M) are derived, respectively, by the fusion of chromosomes 20 and 22, 22 and unknown DNA, and 21 and 22. (B) Comparison of the distribution on human chromosomes of 4q35.2 and Yp11.2 sequences. FISH hybridization analyses were carried out using human BACs AF146191 (4q35.2) and AC006987 (Yp11.2) on human metaphase chromosome spreads. Only chromosomes showing consistent hybridization signals are shown and their corresponding numbers are reported above. All the probes essentially marked the p arm of all acrocentric chromosomes and chromosome 20cen.

To define molecularly the features of sequences at the boundary of human rDNA, we screened a human genomic library with the 28S rDNA probe. Most of the isolated clones were uninformative, either because of the absence of databank sequences or because of the ambiguity of the sequenced ends. However, the sequenced ends (Accession Nos. AQ351037 and AQ351040) of a 28S-positive clone (RP11-119O6) showed 100% similarity with the intergenic sequence (IGS) of the rDNA unit (U13369) and with one fully sequenced but unmapped genomic clone (CR786580) (Supplementary Fig. 1). Interestingly, this clone (with a total length of 195 kb) contained approximately 120 kb from the 4q35.2 region, including *FRG1*, 15 kb from Yp11.2, and other sequences enriched in interspersed repeats and ESTs (Supplementary Fig. 1). The 4q35.2 duplication partially overlapped that found at the MMU rDNA boundary, whereas Yp11.2 sequences differed from those detected within the 50J16 MMU BAC. No 10ptel sequences were detected.

## Discussion

This paper tracked the evolutionary history of DNA sequences linked to the ribosomal DNA cluster in *M. mulatta*. In this species, the flanking DNA is constituted by a patchwork of sequences corresponding to subregions of human Yp11.2, 4q35.2, and 10p15.3. This sequence arrangement was confirmed by the in silico analysis of the MMU genomic draft at UCSC (January 2006) (http://www.genome.UCSC.edu).

Previous results demonstrated that, in humans, the prototypes of BSR sequences were embedded into a duplication preferentially localized on the p arm of acrocentrics and on Yp11.2. Conversely, in the macaque unique or rarely duplicated

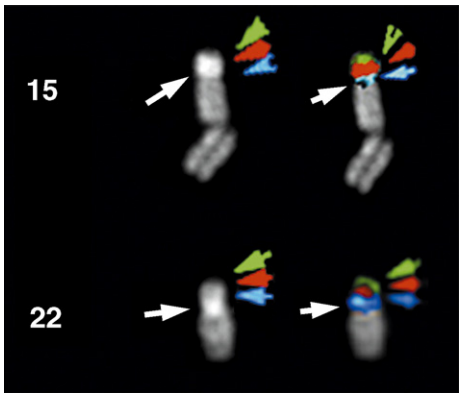


Fig. 4. Relative localization on human acrocentric chromosomes of Yp11.2, 4q35.2, and rDNA sequences. The relative localization was derived by FISH cohybridization with human AF146191 (4q35.2) (red signals), AC006987 (Yp11.2) (blue signals), and dJ-1174A5 (rDNA) (green signals) probes to stretched metaphase chromosome spreads from monochromosomal somatic cell hybrids carrying chromosome 15 or 22 as the only human derivative. The arrows indicate the relative positions on the acrocentric p11 cytogenetic band of 4q35.2 (red), Yp11.2 (blue), and rDNA (green) sequences. White arrows indicate the position of the centromere. On the left of each hybridized chromosome is presented the corresponding DAPI staining.

Yp11.2-like sequences lack the BSR region and are located in the single NOR locus [13]. This observation is now confirmed by the analysis of two MMU BACs (CH250-26D6 and CH250-50J16) that map exclusively on both sides of the NOR on macaque chromosome 13 and contain the ancestral form of the duplication, devoid of the BSR sequences.

In MMU, the prototype of human Yp11.2 duplication is linked to sequences deriving from 4q35.2 and 10p15.3 ancestral regions. This composite DNA shows several peculiar features, including the absence of blocks of repetitive sequences (i.e., alphoid DNA) and the occurrence of a high concentration of interspersed repeats (SINE and LINE). The region also includes at least three genes or pseudogenes (*FRG1, FRG2,* and *TUB4q*) and several ESTs. Thus, in the macaque genome the junction between rDNA and non-rDNA is characterized by a sequence organization unique to that region and resembling a potentially transcribed region. The analysis of *C. aethiops* and *Pr. cristata* suggests that this organization could be shared by all OWM species. In both species 50J16 sequences, indeed, showed a unique location at both boundaries of the NOR.

The data on OWMs allowed us to hypothesize the evolutionary history of the rDNA–non-rDNA transition. In the three analyzed species, chromosomes MMU-13, PCR-15, and CAE-19, which carry the single rDNA array, are derived by the fusion of the chromosome homologous to human chromosome 22 with chromosome 20 in MMU, chromosome 21 in PCR, and DNA of unknown origin in CAE, respectively. In the hypothesized Catarrhini ancestral karyotype, chromosomes homologous to human 20, 21, and 22 are independent entities (http://www.biologia.uniba.it/primates). Interestingly, no 50J16 hybridization signals were detected on the PCR chromosome homologous to human chromosome 20 or on chromosome 2 of CAE (resulting from the fusion of chromosomes homologous to human 21 and 20). Thus, it can be hypothesized that the Catarrhini ancestral chromosome 22 carried the single rDNA array flanked on both sides by a region similar to 50J16 DNA.

Similar to OWM, HLA also showed a specific location of 50J16 sequences around the single NOR of the species. However, in great apes and humans MMU 50J16 sequences underwent duplication and spreading preferentially involving the acrocentric chromosomes. Thus, this spreading can be considered a recent evolutionary event that took place in the ancestor of the great ape–human lineage. In PPY, PTR, and HSA the distribution of 50J16 sequences strongly paralleled the evolutionary dispersal of rDNA clusters. In the GGO the number of chromosomes carrying MMU 50J16 sequences greatly exceeded that bearing rDNA arrays [17]. However, in the latter species the two acrocentrics carrying rDNA (i.e., chromosomes 21 and 22) showed 50J16 hybridization intensities greater than those of the chromosomes not carrying rDNA. These results allow one to hypothesize a role for the duplicative transposition of 50J16 sequences in the evolutionary origin of the multiple rDNA loci in the hominoid species. According to the model of duplicative transposition proposed by Samonte and Eichler [16], in OWM the boundary of rDNA might have functioned as an acceptor locus for sequences located in separate donor loci (i.e., 10p and 4q), thus originating the derived patchwork of sequences (10p/4q/Yp/rDNA). A single colocalization of 50J16 and rDNA sequences was also exhibited by HLA. After the separation of lesser apes from the great ape–human lineage, the 50J16 locus developed into a master donor locus for duplicative transposition to daughter loci, allowing the concomitant spreading of rDNA. In this regard, the gorilla genome shows a spreading of the duplication very similar to that of the orang-utan. However, only GGO chromosomes 21 and 22 bearing rDNA show a consistent presence of the duplication, while the other loci, not carrying rDNA, show a great reduction of 50J16 sequences. These additional loci can be interpreted as evolutionary remnants of previous rDNA localizations, which have undergone a secondary loss of both ribosomal genes and flanking sequences. The demonstration of this hypothesis requires the analysis of the gorilla nucleotide sequences (when available) of these chromosomal regions to identify rDNA signs. In this regard, the analysis of the human genome databank corroborates this interpretation since Yp11.2 sequences, part of which are contained within the MMU rDNA boundary, show the occurrence of a short stretch of ribosomal DNA, thus suggesting a previous linkage with the NOR region.

After the initial burst dispersal of 50J16 and rDNA sequences, which occurred in a great apes ancestor, low-copy sequences showed evolutionary conservation near rDNA loci from PPY to HSA. The fixing of potentially transcribed DNA and interspersed repeats in the vicinity of the rDNA array is intriguing; the potential functionality of this DNA might be that of chromatin structuring finalized to ribosomal genes transcription [18]. However, as derived from the analysis of the human genome, the region at the boundary of rDNA, although maintaining the general feature of low-copy repeats, underwent a marked sequence reorganization. Considering Yp11.2 sequences (AC006987), the evolutionary remodeling included the insertion of the BSR prototype [13] and the under-

representation of 4q35.2 sequences. Furthermore, on the p11 band of human acrocentric chromosomes, large blocks of Yp11.2-like sequences are located more proximal to the centromere than 4q35.2 duplications. In addition, the analysis of a human BAC (CR786580) in linkage with the rDNA array revealed a high enrichment of the 4q35.2 duplication, a very low representation of Yp11.2 sequences, and the absence of 10p sequences. The 4q35.2 duplication partially resembled that found at the rDNA boundary in the macaque, whereas Yp11.2 sequences totally differed. Thus, evolutionary events have certainly modified the ancestral sequence organization of the MMU rDNA boundary, but its general feature has not substantially changed (i.e., 4q35.2 and Yp11.2 low-copy repeats). These chromosomal rearrangements might have been originated by nonallelic homologous recombination facilitated by the duplicated nature of the DNA blocks [16]. In this context, it is important to note that also the sequences adjacent to human rDNA on the p13 band show similarities to those characterized in the present paper, in that they are constituted by low-copy or unique potentially transcribed DNA and interspersed repeats [2,3]. Furthermore, these sequences are also shared by all the acrocentrics and evolutionarily conserved from OWM to human. Thus, previous and present results strongly support the conservation during evolution of the colocalization between rDNA and low-copy-number DNA sequences.

In summary, our results provide additional information on the organization and evolution of the short arm of human acrocentric chromosomes, regions not included in the present human genome databank. By this study we observed that the cospreading of Yp11.2/4q35.2 sequences and rDNA clusters occurred in an ancestor of the great ape–human lineage [11,13], by a mechanism hypothetically based on the duplicative transposition of unique or low-copy DNA. The evolutionary fixing of such sequences near the rDNA of OWM and hominoids adds a potential functionality to this sequence organization, probably reflecting the regulation of the rDNA locus.

## Materials and methods

### *Screening of genomic libraries*

Genomic libraries were obtained from BAC/PAC Resources, Children's Hospital, Oakland Research Institute (HSA, RP11 BAC library, segment 1; MMU, CHORI-250 BAC library, segment 1). High-density arrayed BAC filters were hybridized according to the instructions provided by BAC/PAC Resources (http://bacpac.chori.org/highdensity.htm) with $^{32}$P-labeled human probes. DNA probes were as follows: probe 2* (326 bp) in Fig. 1 and Supplementary Table 1, derived from BAC AC006987 (chromosome Yp11.2) after repeat masking using the software Repeat Masker Web server (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker), and corresponding to a region localized downstream (147844–148171) of the BSR sequence and probe LS6BB, containing the 28S rDNA sequence [14].

### *DNA isolation and Southern blot hybridization*

BAC clones were grown in LB medium supplemented with chloramphenicol. DNA was purified by the Sigma PhasePrep BAC kit (Sigma–Aldrich, USA), digested with different restriction enzymes (Biolabs, USA), fractionated by agarose gel electrophoresis, blotted onto nylon filters (Hybond N+, Amersham, UK), and hybridized with $^{32}$P-labeled probes: alphoid DNA, rDNA/18S (149 bp), and 28S rDNA (clone LS6BB). Molecular hybridizations were performed in 2× SSC at 60°C overnight and filters were washed at 60°C in 1× SSC, twice for 20 min. Hybridization signals were quantified by phosphoimaging (Typhoon 9200, Amersham).

### *Polymerase chain reaction, cloning, and sequencing*

Polymerase chain reaction with the different DNA templates was carried out using the primer pairs listed in Supplementary Table 1 and derived, respectively, from the human genomic DNA sequence U13369 (rDNA), AC006987 (Yp11.2), AF146191 (4q35.2), and AL359957 (10p15.3). Inter-*Alu* polymerase chain reaction was carried out as previously described [15]. Polymerase chain reaction mixtures of 25 μl included 100 ng of DNA, 0.4 μM each primer, 0.2 mM dNTP mix, and 1 U of Red *Taq* DNA polymerase in standard reaction buffer (Sigma–Aldrich). Polymerase chain reaction conditions generally consisted of one preheating cycle at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30–60 s, annealing at 46–65°C for 30 s, and extension at 72°C for 30 s to 2 min.

Inter-*Alu* polymerase chain reaction products were subcloned into the pGEM-T easy vector (Promega, Italy) and sequenced on both strands by the Big Dye terminator 3.1 system (Applied Biosystems, Norwalk, CT, USA) following the manufacturer's instructions. T7/Sp6 BAC ends were sequenced by the Big Dye terminator 3.1 system as described by BAC/PAC Resources (http://bacpac.chori.org/cyclesere.htm). All fluorescent traces were analyzed using the Applied Biosystem Model 3100 DNA sequencing system (Applied Biosystem). DNA sequence analysis was performed by DNASTAR software and by the NCBI facilities (http://www.ncbi.nlm.nih.gov). Newly derived sequences are available at http://www.ncbi.nlm.nih.gov/, under Accession Nos. DQ124861 (CH250-26D6/Sp6), DQ124862 (CH250-26D6/T7), DX571400 (CH250-26D6/AluPCR), DX571397 (CH250-26D6/AluPCR), DQ124866 (CH250-50J16/Sp6), DQ124867 (CH250-50J16/T7), DX571396 (CH250-50J16/AluPCR), DX571398 (CH250-50J16/AluPCR), DX571399 (CH250-50J16/AluPCR) (see Supplementary Table 1).

### *Fluorescence in situ hybridization*

Metaphase chromosome spreads were obtained by standard methods from peripheral blood lymphocytes of normal human donors or from lymphoblastoid or fibroblast primate cell lines of *M. mulatta, C. aethiops, Pr. cristata, H. lar, Po. pygmaeus, G. gorilla,* and *Pan troglodytes* (http://www.biologia.uniba.it/primates/cell_lines.html) and from human monochromosomal somatic cell hybrids carrying chromosome 15 or 22 as the only human derivative (http://www.biologia.uniba.it/rmc/). Stretched metaphase chromosomes were prepared by incubating cells for 2 h at 37°C in 10 μg/ml EtBr and 0.2 μg/ml colcemid. Cells were then collected by centrifugation, resuspended in 75 mM KCl, and fixed by methanol:acetic acid (3:1) solution. After centrifugation, the cell pellet was utilized for the metaphase chromosome preparation. Hybridization probes were labeled with cyanine 3 (red), cyanine 5 (blue), or FluorX (green) dCTP by nick-translation, according to the protocol of the manufacturer (Perkin–Elmer or Amersham), and hybridized overnight to chromosomal preparations. Hybridizations were performed in 50% formamide (v/v), 10% dextran sulfate, 2× SSC at 37°C, in the presence of Cot1 human DNA (Gibco BRL). Posthybridization washing was in 50% formamide, 2× SSC at 42°C, followed by three washes in 1× SSC at 60°C (HSA), or in 50% formamide, 1× SSC at 37°C, followed by three washes in 1× SSC at 42°C (MMU, PCR, and CAE). Chromosomes were stained with DAPI (4′,6-diamidino-2-phenylindole). Digital images were captured using a Leica DMRXA epifluorescence microscope equipped with a cooled CCD camera (Princeton Instruments). Fluorescence signals were recorded separately as gray-scale images. Pseudocoloring and merging of images were performed using Adobe PhotoShop software.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2006.05.001.

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